FOURTH EDITION

MOLECULAR CELL BIOLOGY

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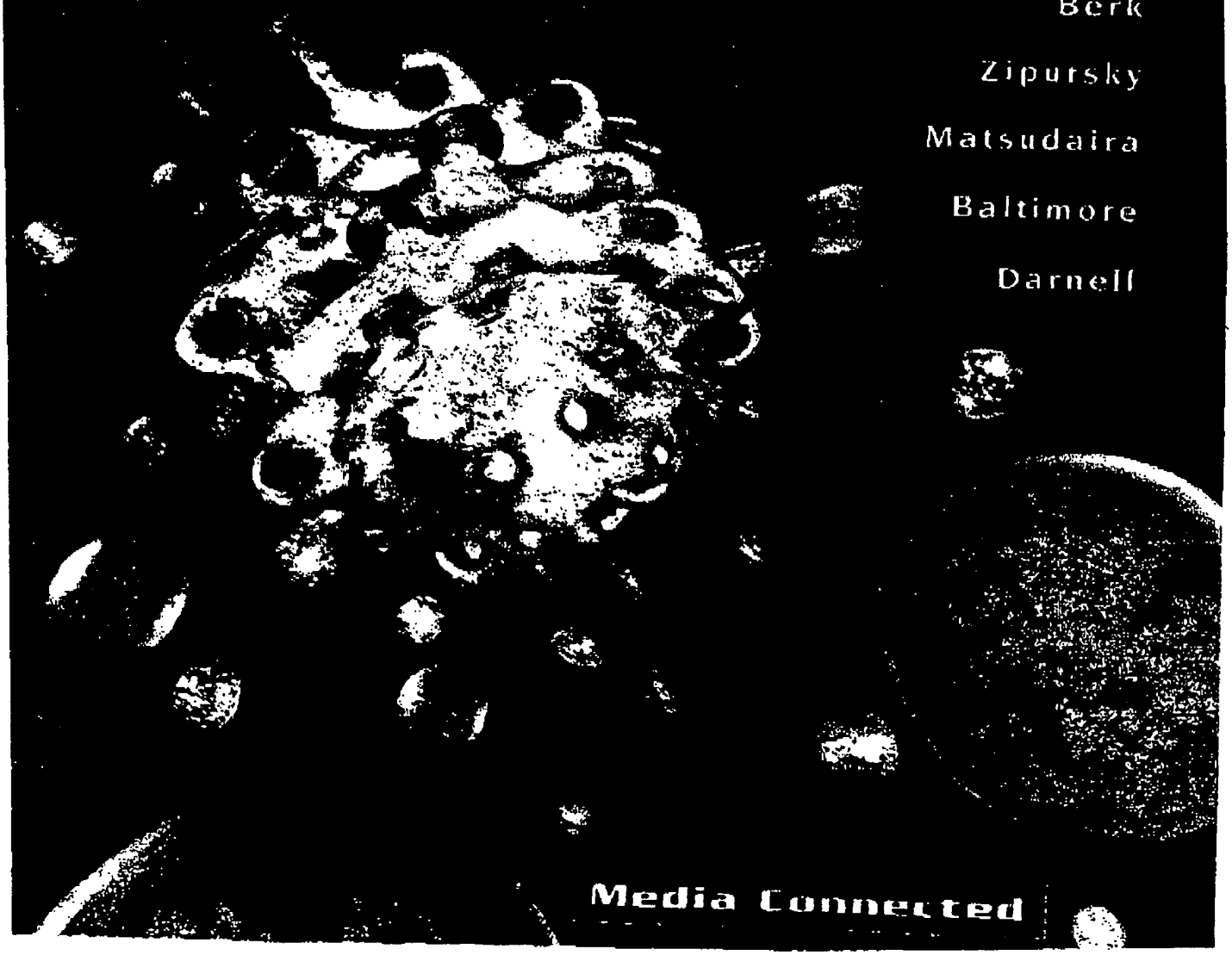

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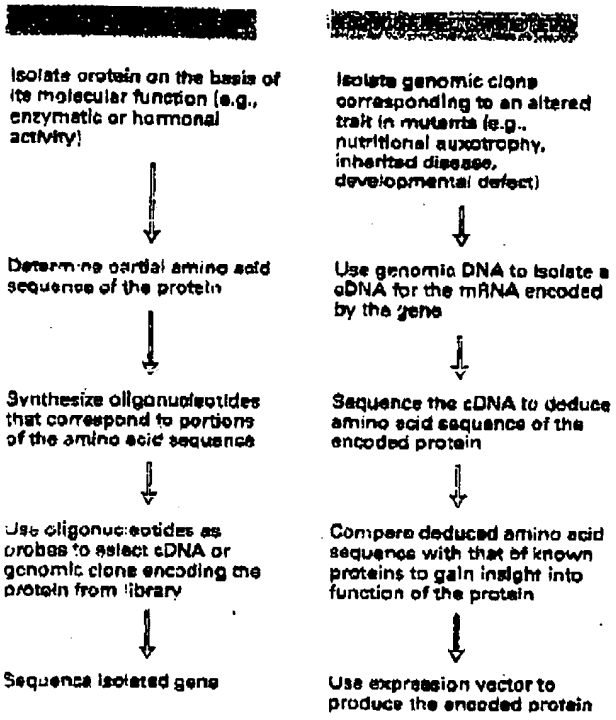

The availability of restriction enzymes also facilitated development of techniques for rapid DNA sequencing in the late 1970s. A long DNA molecule is first cleaved with restriction enzymes into a reproducible array of fragments, whose order in the original molecule is determined. Procedures also were developed for determining the sequence of bases in fragments up to 500 nucleotides long. Thus there was no longer any obstacle to obtaining the sequence of a DNA containing 10,000 or more nucleotides. Suddenly, any DNA could be isolated and sequenced. With the aid of computer-automated procedures for sequencing DNA and for storing, comparing, and analyzing sequence data, scientists will complete sequencing of the entire human genome in the next few years.

In the past, two basic approaches were available for unraveling the molecular basis of complex biological processes: (a) biochemical purification and analysis of a protein based on its functional characteristics (Chapter 3) and (b) classical genetic analysis for the characterization and mapping of genes defined by mutations (Chapter 8). The group of techniques discussed in this chapter, often collectively referred to as *recombinant DNA technology*, provide a link between these two types of experimental strategies, the analysis of proteins and the analysis of genes. Today's molecular cell biologists can begin with an isolated protein and clone the gene that encodes it. They also can reinsert cloned DNA, whether natural, modified, or completely synthetic, into cells and test its biological activity. Alternatively, with the techniques described in Chapter 8, researchers can begin with the concept of a gene identified by the characteristics of a mutant organism and isolate a DNA clone containing the gene. Ultimately, the encoded protein can be produced in sufficient quantities for detailed study. The marriage of biochemical and genetic approaches by recombinant DNA technology provides an enormously powerful strategy for studying the role of particular proteins in cellular processes. In this chapter, we describe the various recombinant DNA techniques that permit this fruitful two-pronged approach, which is summarized in the flow diagram on the left.

7.1 DNA Cloning with Plasmid Vectors

The essence of cell chemistry is to isolate a particular cellular component and then analyze its chemical structure and activity. In the case of DNA, this is feasible for relatively short molecules such as the genomes of small viruses. But genomes of even the simplest cells are much too large to directly analyze in detail at the molecular level. The problem is compounded for complex organisms. The human genome, for example, contains about 6×10^9 base pairs (bp) in the 23 pairs of chromosomes. Cleavage of human DNA with restriction enzymes that produce about one cut for every 3000 base pairs yields some 2 million fragments, far too many to separate from each other directly. This obstacle to obtaining pure DNA samples from large genomes has been overcome by recombinant DNA technology. With these methods virtually any gene can be purified, its sequence determined, and the functional regions of the sequence explored by altering it in planned ways and reintroducing the DNA into cells and into whole organisms.

The essence of recombinant DNA technology is the preparation of large numbers of identical DNA molecules. A DNA fragment of interest is linked through standard $3' \rightarrow 5'$ phosphodiester bonds to a vector DNA molecule, which can replicate when introduced into a host cell. When a single recombinant DNA molecule, composed of a vector plus an inserted DNA fragment, is introduced into a host cell, the inserted DNA is reproduced along with the vector, producing large numbers of recombinant DNA molecules that include the fragment of DNA originally linked to the vector. Two types of vectors are most commonly used: *E. coli* plasmid vectors and bacteriophage λ vectors. Plasmid vectors replicate along with their host cells, while λ vectors replicate as lytic viruses, killing the host cell and packaging the DNA into virions (Chapter 6). In this section, the general procedure for cloning DNA fragments in *E. coli* plasmids is described.

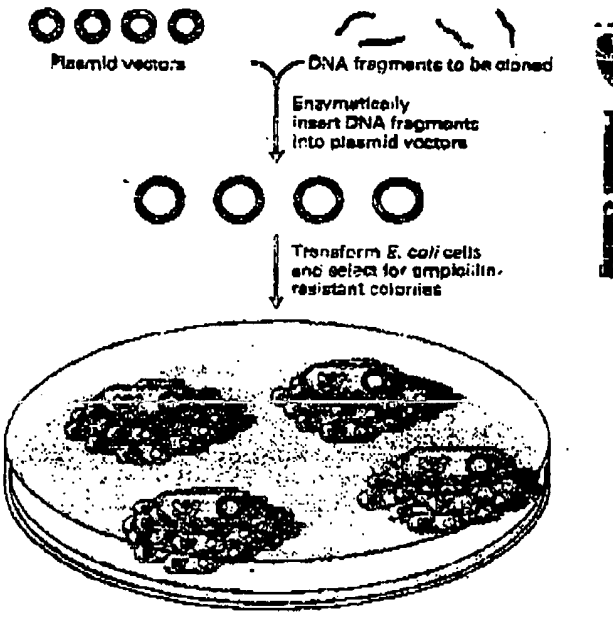

FIGURE 7-4 Isolation of DNA fragments from a mixture by cloning in a plasmid vector. Four distinct DNA fragments, depicted in different colors, are inserted into plasmid cloning vectors, yielding a mixture of recombinant plasmids each containing a single DNA fragment. *E. coli* cells treated with $CaCl_2$ are incubated with the mixture of recombinant plasmids and then plated on nutrient agar containing ampicillin. Each colony of transformed, antibiotic-resistant cells that grows (represented by a group of cells) arises from a single cell that took up one or another of the recombinant plasmids; all the cells in a given colony thus carry the same DNA fragment. Overnight incubation of *E. coli* at 37 °C produces visible colonies containing about a million cells. Since the colonies are separated from one another on the culture plate, copies of the DNA fragments in the original mixture are isolated in the individual colonies. Although it's not shown here, the transformed cells contain multiple copies of a given plasmid.

rest of the plasmid DNA and segregates to daughter cells as the colony grows. In this way, the initial fragment of DNA is replicated in the colony of cells into a large number of identical copies. Since all the cells in a colony arise from a single transformed parental cell, they constitute a clone of cells. The initial fragment of DNA inserted into the parental plasmid is referred to as *cloned DNA*, since it can be isolated from the clone of cells.

DNA cloning allows fragments of DNA with a particular nucleotide sequence to be isolated from a complex mixture of fragments with many different sequences. As a simple example, assume you have a solution containing four different types of DNA fragments, each with a unique sequence (Figure 7-4). Each fragment type is individually inserted into a plasmid vector. The resulting mixture of recombinant plasmids is incubated with *E. coli* cells under conditions that facilitate transformation; the cells then are cultured on antibiotic selective plates. Since each colony that develops arose from a single cell that took up a single plasmid, all the cells in a colony harbor the identical type of plasmid characterized by the DNA fragment inserted into it. As a result, copies of the DNA fragments in the initial mixture are isolated from one another in the separate bacterial colonies. DNA cloning thus is a powerful, yet simple method for purifying a particular DNA fragment from a complex mixture of fragments and producing large numbers of the fragment of interest.

Restriction Enzymes Cut DNA Molecules at Specific Sequences

To clone specific DNA fragments in a plasmid vector, as just described, or in other vectors discussed in later sections, the fragments must be produced and then inserted into the vector DNA. As noted in the introduction, restriction enzymes and DNA ligases are utilized to produce such recombinant DNA molecules.

Restriction enzymes are bacterial enzymes that recognize specific 4- to 8-bp sequences, called *restriction sites*, and then cleave both DNA strands at this site. Since these enzymes cleave DNA within the molecule, they are also called *restriction endonucleases* to distinguish them from exonucleases, which digest nucleic acids from an end. Many restriction sites, like the *Eco*RI site shown in Figure 7-5a, are short inverted repeat sequences; that is, the restriction-site sequence is the same on each DNA strand when read in the $5' \rightarrow 3'$ direction. Because the DNA isolated from an individual organism has a specific sequence, restriction enzymes cut the DNA into a reproducible set of fragments called restriction fragments (Figure 7-6).

The word *restriction* in the name of these enzymes refers to their function in the bacteria from which they are isolated: a restriction endonuclease destroys (restricts) incoming foreign DNA (e.g., bacteriophage DNA or DNA taken up during transformation) by cleaving it at all the restriction sites in the DNA. Another enzyme, called a *modification enzyme*, protects a bacterium's own DNA from cleavage by modifying it at or near each potential cleavage site. The modification enzyme adds a methyl group to one or two bases, usually within the restriction site. When a methyl group is present there, the restriction endonuclease is prevented from cutting the DNA (Figure 7-5b). Together with the restriction endonuclease, the methylating enzyme forms a restriction-modification system that protects the host DNA while it destroys foreign DNA. Restriction enzymes have been purified from several hundred different species of bacteria, allowing DNA molecules to be cut at a large number of different sequences corresponding to the recognition sites of these enzymes (Table 7-1).

TABLE 7-1 Selected Restriction Endonucleases and Their Restriction-Site Sequences

Source Microorganism	Enzyme*	Recognition Site (↓)†	Ends Produced
Arthrobacter luteus	*AluI*	AG↓CT	Blunt
Bacillus amyloliquefaciens H	*BamHI*	G↓GATCC	Sticky
Escherichia coli	*EcoRI*	G↓AATTC	Sticky
Haemophilus gallinarum	*HgaI*	GACGC+5↓	‡
Haemophilus influenzae	*HindIII*	A↓AGCTT	Sticky
Haemophilus parahaemolyticus	*HphI*	GGTGA+8↓	‡
Nocardia otitiscaviarum	*NotI*	GC↓GGCCGC	Sticky
Staphylococcus aureus 3A	*Sau3AI*	↓GATC	Sticky
Serratia marcesens	*SmaI*	CCC↓GGG	Blunt
Thermus aquaticus	*TaqI*	T↓CGA	Sticky

*Enzymes are named with abbreviations of the bacterial strains from which they are isolated; the roman numeral indicates the enzyme's priority of discovery in that strain (for example, AluI was the first restriction enzyme to be isolated from Arthrobacter luteus).

†Recognition sequences are written 5'→3' (only one strand is given), with the cleavage site indicated by an arrow. Enzymes producing blunt ends cut both strands at the indicated site; those producing sticky ends make staggered cuts, with cleavage occurring between the same nucleotides in each strand as shown in Figure 7-5a.

‡The cleavage sites for HphI and HgaI occur several nucleotides away from the recognition sequence. HgaI cuts five nucleotides 3' to the GACGC sequence on the top strand and ten nucleotides 5' to the complementary CTGCG sequence on the bottom strand. HphI cuts eight nucleotides 3' to the CGTGA sequence on the top strand and seven nucleotides 5' to the complementary CCACT sequence on the bottom strand.

SOURCE: R. J. Roberts, 1988, *Nucl. Acids Res.* 16(suppl):271.

During in vivo DNA replication, DNA ligase catalyzes formation of 3' → 5' phosphodiester bonds between the short fragments of the discontinuously synthesized DNA strand at a replication fork (see Figure 4-16). In recombinant DNA technology, purified DNA ligase is used to covalently join the ends of restriction fragments in vitro. This enzyme can catalyze the formation of a 3' → 5' phosphodiester bond between the 3'-hydroxyl end of one restriction-fragment strand and the 5'-phosphate end of another restriction-fragment strand during the time that the sticky ends are transiently base-paired (Figure 7-7). When DNA ligase and ATP are added to a solution containing restriction fragments with sticky

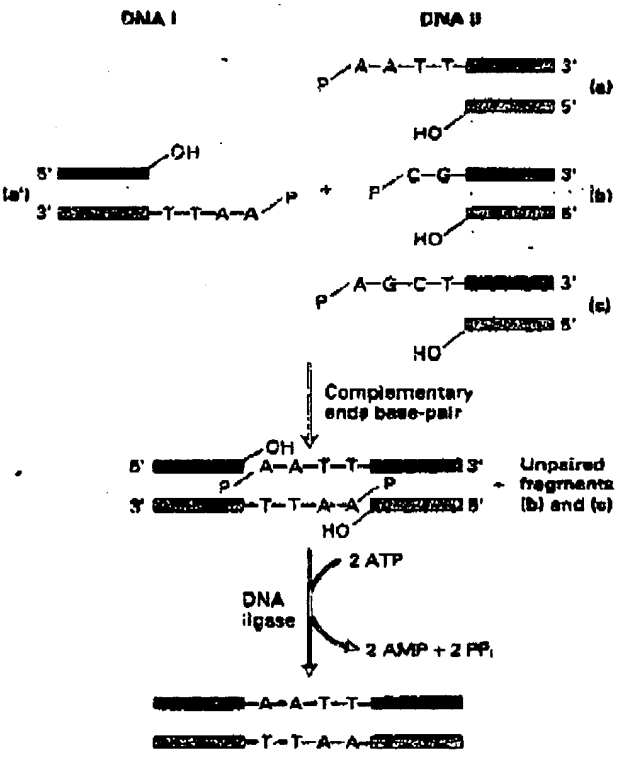

FIGURE 7-7 Ligation of restriction fragments with complementary sticky ends. In this example, *EcoRI* fragments from DNA I (left) are mixed with several different restriction fragments, including *EcoRI* fragments, produced from DNA II (right). The short DNA sequences composing the sticky ends of each fragment type are shown. The complementary sticky ends on the two types of *EcoRI* fragments, (a') and (a), can transiently base-pair, whereas the *TaqI* fragments (b) and *HindIII* fragments (c) with noncomplementary sticky ends do not base-pair to *EcoRI* fragments. The adjacent 3'-hydroxyl and 5'-phosphate groups (red) on the base-paired fragments then are covalently joined (ligated) by T4 DNA ligase. One ATP is consumed for each phosphodiester bond (red) formed.

receptor Any protein that binds a specific extracellular signaling molecule (ligand) and then initiates a cellular response. Receptors for steroid hormones, which diffuse across the plasma membrane, are located within the cell; receptors for water-soluble hormones, peptide growth factors, and neurotransmitters are located in the plasma membrane with their ligand-binding domain exposed to the external medium.

receptor tyrosine kinase (RTK) Member of an important class of cell-surface receptors whose cytosolic domain has tyrosine-specific protein kinase activity. Ligand binding activates this kinase activity and initiates intracellular signaling pathways. (Figure 20-23)

recessive In genetics, referring to that allele of a gene that is not expressed in the phenotype when the dominant allele is present. Also refers to the phenotype of an individual (homozygote) carrying two recessive alleles. (Figure 8-1)

recombinant DNA Any DNA molecule formed by joining DNA fragments from different sources. Commonly produced by cutting DNA molecules with restriction enzymes and then joining the resulting fragments from different sources with DNA ligase.

recombination Any process in which chromosomes or DNA molecules are cleaved and the fragments are rejoined to give new combinations. Occurs naturally in cells as the result of the exchange (crossing over) of DNA sequences on maternal and paternal chromosomes during meiosis; also is carried out in vitro with purified DNA and enzymes.

reduction Gain of electrons by an atom or molecule as occurs when hydrogen is added to a molecule or oxygen is removed. The opposite of oxidation.

reduction potential The voltage change when an atom or molecule gains an electron.

replication fork See growing fork.

replication origin Unique DNA segments present in an organism's genome at which DNA replication begins. Eukaryotic chromosomes contain multiple origins, whereas bacterial chromosomes and plasmids often contain just one.

replicon Region of DNA served by one replication origin.

resolution The minimum distance that can be distinguished by an optical apparatus; also called *resolving power*.

respiration General term for any cellular process involving the uptake of O_2 coupled to production of CO_2.

restriction enzyme (endonuclease) Any enzyme that recognizes and cleaves a specific short sequence, the restriction site, in double-stranded DNA molecules. These enzymes are widespread in bacteria and are used extensively in recombinant DNA technology. (Table 7-1 and Figure 7-51)

restriction fragment A defined DNA fragment resulting from cleavage with a particular restriction enzyme. These fragments are used in the production of recombinant DNA molecules and DNA cloning.

restriction point The point in late G_1 of the cell cycle at which mammalian cells become committed to entering the S phase and completing the cycle even in the absence of growth factors.

retrotransposon Type of eukaryotic mobile DNA element whose movement in the genome is mediated by an RNA intermediate and involves a reverse transcription step. See also transposon.

retrovirus A type of eukaryotic virus containing an RNA genome that replicates in cells by first making a DNA copy of the RNA. This proviral DNA is inserted into cellular chromosomal DNA, and gives rise to further genomic RNA as well as the mRNAs for viral proteins. (Figure 6-22)

reverse transcriptase Enzyme found in retroviruses that catalyzes synthesis of a double-stranded DNA from a single-stranded RNA template. (Figure 9-16)

ribosomal RNA See rRNA.

ribosome A large complex comprising several different rRNA molecules and more than 50 proteins, organized into a large subunit and small subunit; the site of protein synthesis. (Figures 4-32 and 4-34)

ribozyme An RNA molecule or segment with catalytic activity.

RNA (ribonucleic acid) Linear, single-stranded polymer, composed of ribose nucleotides, that is synthesized by transcription of DNA or by copying of RNA. The three types of cellular RNA—mRNA, rRNA, and tRNA—play different roles in protein synthesis.

RNA editing Unusual type of RNA processing in which the sequence of a pre-mRNA is altered.

RNA polymerase An enzyme that copies one strand of DNA or RNA (the template strand) to make the complementary RNA strand using as substrates ribonucleoside triphosphates.

RNA processing Various modifications that occur to many but not all primary transcripts to yield functional RNA molecules.

RNA splicing A process that results in removal of introns and joining of exons in RNAs. See also spliceosome. (Figure 11-16)

rRNA (ribosomal RNA) Any one of several large RNA molecules that are structural and functional components of ribosomes. Often designated by their sedimentation coefficient: 28S, 18S, 5.8S, and 5S rRNA in higher eukaryotes.

S (synthesis) phase See cell cycle.

sarcoma A malignant tumor derived from connective tissue.

sarcomere Repeating unit of a myofibril in striated muscle that extends from one Z disk to an adjacent one and shortens during contraction. (Figure 18-27)

sarcoplasmic reticulum Network of membranes that surrounds each myofibril in a muscle cell and sequesters Ca^2 ions. Stimulation of a muscle cell induces release of Ca^2 ions into the cytosol, triggering coordinated contraction along the length of the cell. (Figure 18-31)

Schwann cell Type of glial cell that forms the myelin sheath around axons in the peripheral nervous system.

second messenger An intracellular signaling molecule whose concentration increases (or decreases) in response to binding of an extracellular ligand to a cell-surface receptor. Examples include cAMP, Ca^2, diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP_3). (Figure 20-4)

secondary structure In proteins, local folding of a polypeptide chain into regular structures including the α helix, β sheet, and U-shaped turns and loops.

secretory vesicle Small membrane-bound organelle containing molecules destined to be released from the cell.

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